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Abstract

Apple trees cv. 'Golden Delicious' and 'York' were sprayed from bloom to fruit maturity with different products to evaluate the effect of pre-harvest treatments on fruit quality, including insect/disease damage and physicochemical fruit traits. Apple trees were assigned to five treatments: unsprayed (control), holistic solution (foliar nutrients and probiotics), insecticides, antimicrobials (fungicides and antibiotics), and a combination of antimicrobials + insecticides. The treatments started soon after bloom and were carried out every two weeks until fruit were ready to harvest.

Diseases such as sooty blotch (complex of several fungi) and flyspeck (*Zygophiala jamaicensis* Mason) were the major source of damage on fruits. 'Golden Delicious' trees had a higher percentage of undamaged fruit than 'York', but all trees had some percentage of damaged fruit. Damage was most severe in the control (unsprayed) and insecticide treatments, intermediate in the holistic treatment, and much lower in the antimicrobial and antimicrobial + insecticide treatments ($p<0.003$ for all comparisons). There was also a significant interactive effect ($p<.0001$) of cultivars and pre-harvest spray treatment on apple fruit mass. For both cultivars there was a strong effect of spray treatment on size, with larger apples produced in the antimicrobial and antimicrobial + insecticide treatments, but when apple trees were not sprayed (control) or sprayed with holistic and insecticides treatments, the fruit mass was higher in 'Golden Delicious' than 'York'. 'Golden Delicious' trees produced 1.4-fold heavier and bigger fruits compared to 'York' and 'Golden Delicious' fruit were more mature than 'York' at harvest. Pre-harvest treatments also affected other quality parameters of apple fruit, such as soluble solids content (SSC) and starch-iodine index. Using partial least squares discriminant analysis (PLS-DA), 'Golden Delicious' fruit could be well classified according to the holistic, antimicrobial, and antimicrobial + insecticide treatments. Control and insecticide samples clustered together, indicating similarities between fruit quality. Overall, pre-harvest spray treatment affected the quality of 'Golden Delicious' and 'York' apples, mainly the fruit mass and disease infection.

Keywords: *Malus* x *domestica* Borkh., fungicides, antibiotics, insecticides, probiotics, physicochemical composition.

1. Introduction

Apple is the fourth most consumed fruit in the world and the apple annual production in 2016 reached 89,329,179 tons (FAOSTAT, 2018). Apples are popular with consumers due to their convenience and nutritional value, and the iconic image of the apple as a health-promoting fruit has stimulated extensive research surrounding the health benefits of apple phytochemicals (Boyer and Liu, 2004). Various quality parameters, including pest damage and other traditional quality parameters such as soluble solids content (SSC) and firmness, are all very important attributes for apple acceptance (McCluskey et al., 2013).

According to Abbott (1999), quality is a term that implies “the degree of excellence of a product or its suitability for a particular use”. It is a flexible concept and can involve various properties or characteristics. The quality of fresh produce comprises many attributes, such as appearance (size, shape, color, gloss, presence of defects and decay), texture (firmness, crispness, juiciness, mealiness, and toughness), flavor (sweetness, acidity, astringency, aroma, and off-flavors), and nutritive value (vitamins, minerals, dietary fiber, phytonutrients) (Kader 2001). According to Vanoli and Buccheri (2012), consumers first evaluate produce by its appearance (presence of damage, color, size and shape) and then its eating quality. Although appearance typically determines the purchase of produces, flavor is an important quality parameter for apple consumers' acceptance (Aprea et. al., 2012) and consumers satisfaction will influence the repeat purchases (Kader, 2001). Regarding flavor, the sweet and acid taste of apple fruits are key sensory attributes for consumer preference (Jaeger et al., 1998) and SSC might be used as a predictor of sweetness while the acid taste may be predicted based on the titratable acidity (Harker et al., 2002). The relationships between SSC and titratable acidity (TA) commonly called ratio (Kader, 2001) presents good relationships between apple fruit quality and consumer acceptability, therefore it is an important quality attribute for apple evaluation.

Commercial quality standards for apples are based on aspects such as size, color, integrity, and presence and/or absence of defects (Musacchi and Serra, 2018). Although post-harvest management can affect the quality of apples, various pre-harvest practices can also affect and modify fruit quality attributes. The pre-harvest factors that can affect apple quality might be grouped into genetic (rootstock and cultivars), environmental (soil, light, temperature, humidity, wind), and agronomic (nutrition, irrigation, training system, pruning, crop load/thinning, plant growth regulator, pollination, etc.) factors (Musacchi and Serra, 2018).

Apples, in particular, are heavily attacked by insects and disease and require intensive pre-harvest management to produce marketable fruit (Beers et al., 2003). For a considerable period, chemicals have been used to control pests and diseases. Starting several decades ago, apples have received more pesticides than any other fruit crop in the United States of America (Huffaker and Croft, 1978), a trend that continues today (USDA, 2018). Applying pesticides can have unanticipated effects on various components of fruit quality (Schuphan, 1961), although pesticides can be highly effective at controlling disease and insect pressure. Therefore, pre-harvest spray programs might not just control pest and diseases, but also influence apple quality parameters, such as firmness, SSC, TA and aroma (Róth et al., 2007), and these impacts should be taken in consideration in pre-harvest practices. Many studies can be found comparing the apple quality produced in conventional farming, integrated pest management systems, and organic systems (Weibel et al., 2000; Peck et al., 2006; Róth et al., 2007; Amarante et al., 2008; Jönssson et al., 2010); however, few have specifically examined the effect of pre-harvest spray procedures on fruit quality (Hutcheon et al., 1986; Palmer et al., 2003). Therefore, the objective of this study was to investigate how different pre-harvest spray programs could affect ‘Golden Delicious’ and ‘York’ apple fruit quality.

2. Material and methods

2.1. Plant material

The experiment was carried out at Virginia Polytechnic Institute and State University (Virginia Tech), Kentland Farm, Blacksburg, Virginia, the United States of America (USA). The orchard is located at 37º 11’ 23’’ North and 80° 34’ 35’’ West, 516 meters above sea level. The Köppen climate classification subtype is humid subtropical climate (Cfa).

The apple orchard was 16 years old and the ‘Golden Delicious’ and ‘York’ apples were grafted on M.26 rootstock apples and planted at 2.5 x 4.5 m spacing in a soil classified as Braddock loam, fine, mixed, semi active, mesic Typic Hapludults (Penn et al., 2004). The orchard has historically been managed with conventional spray programs. The orchard was not irrigated, and the fertilization program was the same for both cultivars.

2.2. Experimental setup

During the 2018 summer/fall season, 36 ‘Golden Delicious’ and 33 ‘York’ apple trees were selected for uniformity and divided into five groups, related to the following treatments: 1, control – unsprayed trees, 2, trees sprayed with a ‘holistic’ solution, 3, trees sprayed with insecticides, 4, trees sprayed with antimicrobials (fungicides and antibiotics), and 5, trees sprayed with antimicrobials + insecticides. Details on the components of each treatment are provided in Table 1. The holistic treatment was a combination of products developed and recommended by Phillips (2012) and it is currently commercially marketed from organic grower supply companies (e.g. Fedco Seeds). It includes macronutrients and trace minerals that can act as foliar fertilizers, probiotics and nutrients intended to support microorganisms on the plant surface, and neem oil extracts that can act as botanical insecticides. To our knowledge, this spray has not been evaluated in a scientific context for its effects on fruit quality. The treated apple trees were separated by one guard tree and a buffer row to avoid the effect of spray drift. Apple trees were hand thinned, aiming to remove excessive fruitlets from the plants. The chemical products sprayed were applied on the trees every two weeks beginning at bloom and continuing through mid-September (Table 1).

The experiment was laid down according to a randomized complete block design (RCBD) with two blocks (cultivars – ‘Golden Delicious’ and ‘York’) containing all five treatments (1, control, 2, holistic, 3, insecticides, 4, antimicrobials, and 5, combination antimicrobials + insecticides) with 6-8 replicate apple trees per treatment/cultivars combination. ‘Golden Delicious’ fruit harvest was carried out from October 3rd to 12th and ‘York’ from October 12th to 19th 2018.

2.3. Fruit damage evaluation

At maturity, apple samples were collected for fruit damage evaluation. Fruits from all treatments were harvested, with up to 20 fruits per tree and totaling 684 'Golden Delicious' and 395 'York' fruit. The apple fruit were evaluated according to the following pest and diseases:

Pests. Insect damage was identified following the descriptions reported by Agnello et al. (2006). The most common insect damage was caused by plum curculio beetles (*Conotrachelus nenuphar* Herbst) and the number of fruits with the typical crescent-shaped blemishes were counted and the data transformed using the square root of x+1.

Diseases. The symptoms of the most common diseases were identified following the description of Agnello et al. (2006). The presence of sooty blotch, attributed to a complex of different fungi, and flyspeck (*Zygophiala jamaicensis* Mason), were evaluated using a five-point scale, 0 = undamaged fruit, 1 = < 5%, 2 = 5 – 25%, 3 = 25 – 50%, 4 = 50 – 75%, and 5 = > 75% skin coverage. Cedar apple rust disease (*Gymnosporangium juniperi-virginianae*) was also observed and the number of fruits with the typical pale-yellow pinhead sized spots were counted and the data transformed using the square root of x+1.

2.4. Quality evaluations

At maturity, apple samples were also collected for quality evaluation. Three apples were harvested per plant from all treatments, totaling 207 fruit. The apple fruit were evaluated according to the following quality parameters:

Fruit mass. The mass was determined for all harvested fruit using an analytical balance (Radwag, model AS 60/220-R2, Miami Beach, USA) and the mass results were expressed in grams (g).

Firmness. The pulp firmness was determined using a penetrometer (Fruit Hardness Tester, model FHT-1122, Merit Technology, Shahekou, China) with a 11.0 mm tip. The determinations were performed on each fruit after removing the peel. The results were expressed in Newton (N).

Fruit maturity. The harvest maturity was determined using the Cornell starch-iodine index (Blanpied and Silsby, 1992). To assess maturity, apple fruit were cut, and the slices were placed into iodine solution for 30 seconds and allowed to dry for 20 minutes. The color of the slices was compared to the Cornell starch-iodine chart and the maturity recorded from 1 (immature) to 8 (ripe).

Soluble solids content. Freshly squeezed apple juice was used to determine the soluble solids content (SSC). A handheld refractometer BX-20 (Veegee Analytical Instruments, Kirkland, USA) was used and the SSC was expressed as mass percentage (%) in the solution, A.O.A.C. (1997).

Moisture. The moisture content of the apple pulp was determined by the sample's loss in mass after drying for 70 hours at 65 °C in an oven/incubator (Type 19200, Thermolyne, Thermo Fisher Sci. Inc., Waltham, USA), which allowed samples to reach constant mass (A.O.A.C., 1997).

Dry matter. The dry matter (DM) content was determined using the formula DM = 100 – M, where: DM = dry matter and M = moisture content. DM content was expressed as gram per kilogram (A.O.A.C., 1997).

2.5. Univariate statistical analysis

To compare individual quality parameters among treatments, the data were subjected to analysis of variance (ANOVA) using the PROC GLM procedure of the Statistical Analysis System (SAS, 1999). Treatment and cultivars were included as fixed effects, tree was included as a random effect, and the treatment means were compared using the Tukey's test at a significance level of $p < 0.05$.

2.6. Multivariate statistical analysis

Multivariate analyses were carried out first using the physicochemical traits and then with all quality parameters (including damage) to assess the overall differences among treatments. The discriminant models were developed separately for each cultivar and combining the results of both averaging the data per tree within each pre-harvest treatment as the data was obtained from different fruit numbers.

The data were processed using MATLAB® R2014b software (MathWorks, USA) with PLS Toolbox version 7.9.3 (Eigenvector Research, Inc., USA). The data were auto scaled before analysis. Samples were divided into training (70 %) and test (30 %) sets using the Kennard-Stone uniform sample selection algorithm (Kennard and Stone, 1969). The training set was used for model construction and the test set for final model evaluation. Cross-validation venetian blinds with eight data splits was employed for model optimization.

Initially, principal component analysis (PCA) was employed for exploratory analysis of the data (Bro and Smilde, 2014). Sample classification was then performed using the partial least squares discriminant analysis (PLS-DA) algorithm (Brereton and Lloyd, 2014). The main difference between PLS-DA and PCA is that PLS decomposes the data in an interactive process involving both the experimental observations and category information, therefore generating scores and loadings for both data sets as follows:

$$\mathbf{X} = \mathbf{TP} + \mathbf{E} \quad (01)$$

$$\mathbf{y} = \mathbf{Tq} + \mathbf{f} \quad (02)$$

Where **X** is a matrix containing the experimental observations; **y** is a vector containing the sample's category (e.g., 0/1); **T** is a common scores matrix; **P** is matrix containing the loadings of the experimental observations; **E** are the data residuals; **q** represents the loadings of the category variables; and **f** the category residuals. In PLS-DA, a linear classifier is employed to the predicted PLS response $\hat{\mathbf{y}}$ separating the data into groups, where $\hat{\mathbf{y}}$ is estimated using the regression coefficients **b** as follows:

$$\hat{\mathbf{y}} = \mathbf{Xb} = \mathbf{X}[\mathbf{W}(\mathbf{PW})^{-1}\mathbf{q}] \quad (03)$$

In which **W** is the weight matrix (Brereton and Lloyd, 2014).

One of the most popular measures is the area under the ROC curve (AUC). AUC is a combined measure of sensitivity (Equation 4) and specificity (Equation 5), respectively. AUC is a measure of the overall performance of a diagnostic test and is interpreted as the average value of sensitivity for all possible values of specificity. It can take on any value between 0 and 1, since both the x and y axes have values ranging from 0 to 1.

$$sens\ (\%) = \frac{TP}{TP+FN}\ x\ 100 \qquad (04)$$

$$spec\ (\%) = \frac{TN}{TN+FP}\ x\ 100 \qquad (05)$$

TP is true positive, FP is false positive, TN is true negative and FN is false negative.

3. Results

3.1. Fruit damage evaluation

All fruit presented some sort of damage (disease and/or insect) and it was more severe in the control (unsprayed), holistic and insecticide treatments, which resulted in 0.0% undamaged fruit. The antimicrobial treatments resulted in 15.0% and 6.67% undamaged fruit for 'Golden Delicious' and 'York', respectively. This percentage was increased to 48.57% when 'Golden Delicious' trees were sprayed with the antimicrobial + insecticide treatment, compared to only 10.71% for 'York' (Figure 1). In the ANOVA assessing how pre-harvest spray treatment and cultivars affected the percentage of undamaged fruit, there was a significant interaction between cultivars and treatment ($F_{9,62}$=6.32, p=0.0003), a significant difference among treatments ($F_{9,62}$=29.84, p=<.0001) and a significant difference between cultivars ($F_{9,62}$=8.19, p=0.0060; Figure 1). 'Golden Delicious' presented the highest average percentage of undamaged fruit (12.71%) compared to 'York' (4.11%). Based on the interaction between treatment and cultivars, differences among treatments were also analyzed separately for each cultivar (Figure 1). The major difference between cultivars was that in 'Golden Delicious' apples the antimicrobial + insecticide treatment was more clearly distinguishable from the antimicrobial only treatment, resulting in a higher percentage of undamaged fruit (Figure 1).

Fruits from both cultivars were severely infected by sooty blotch and flyspeck, but the severity varied among treatments and cultivars (Figure 2). Infection was strongly reduced when plants were sprayed with antimicrobials or antimicrobials + insecticides (Table 2). There was also a significant reduction in infection severity in the holistic treatment compared to the insecticide treatment and controls (Table 2). In addition, the severity of both flyspeck and sooty blotch was higher in 'York' apples compared to 'Golden Delicious' (Table 2). Cedar apple rust disease was also present on leaves and fruits and caused minor damages. There were no differences in this disease among treatments, but 'York' fruit were more affected than 'Golden Delicious' (Table 2).

Regarding insect damage, the most common blemish was caused by plum curculio beetles, but no significant differences ($p>0.1526$) were observed between cultivars or among pre-harvest treatments ($p>0.0688$; Table 2).

3.2. Fruit quality evaluation

Apple fruit mass was significantly affected by the pre-harvest treatments, the cultivars, and the interaction between treatment and cultivars (Table 3; Figure 3). Both cultivars produced heavier fruit in the antimicrobial + insecticide treatment, intermediate size fruit in the antimicrobial treatment, and the smallest fruits in the control, holistic, and insecticide treatments (Figure 3); however, the differences among treatments were more pronounced for 'York' apples than for 'Golden Delicious'. 'Golden Delicious' trees also produced 1.4-fold heavier and bigger fruits ($110.61^{\pm}22.80$ g) compared to 'York' ($76.67^{\pm}40.42$ g).

For the physicochemical aspects of fruit quality, the different spray treatments affected only the SSC and maturity (Table 3). The highest SSC was observed in apples from the holistic spray treatment and the lower starch-iodine index was obtained in apples sprayed with antimicrobials (Table 3). In addition, most of these variables differed between the two cultivars (Table 3). At harvest, 'Golden Delicious' apples were more mature than 'York' based on the starch-iodine index (Table 3). 'Golden Delicious' fruit also had higher mean SSC, fruit firmness, and dry-matter content, but lower moisture content than 'York' fruit (Table 3).

3.3. Multivariate analysis

3.3.1. Physicochemical discrimination models

Using separate PCAs for 'Golden Delicious' and 'York' apple samples, a tendency was observed of separation between two clusters, one representing the control, holistic, and insecticide treatments and another representing the antimicrobial and antimicrobial + insecticide treatments (Figure 1S-A, Figure 1S-C). Samples mainly separated along PC2, which was associated with increasing fruit maturity and decreasing fruit mass (Figure 1S-B, Figure 1S-D). However, a clear separation between clusters was not observed as superposition of samples was observed along PC1 and PC2.

Better separation between the two clusters was obtained by using PLS-DA (Table 1S). For 'Golden Delicious' samples, the antimicrobials had the best discriminatory values with an area under the curve (AUC) of 0.97 (almost perfect classification), indicating that this class was highly different from the others. The holistic, insecticide and the combination of antimicrobial + insecticides have fair classification results (AUC ranging from 0.69 to 0.76). The control samples had the worst classification result (AUC=0.57). For 'York' samples, the classification performance was slightly better. The antimicrobials treatment still had the best classification (AUC=0.89), and the AUC values improved for the control, insecticide, and the combination of antimicrobial + insecticides.

The discriminant function (DF) and PLS-DA coefficients for 'Golden Delicious' and 'York' samples can be seen in Figure 2S. The fruit mass and fruit maturity in 'Golden Delicious' samples were the main parameters associated with the good classification of antimicrobial and insecticide pre-harvest sprayed samples; while for the holistic treatment, SSC, moisture, and DM were the most important parameters (Figure 2S-B). For 'York' samples, mass and fruit maturity were also the main parameters responsible for all class differentiations. SSC and moisture had little influence, except for the holistic and control samples; and firmness influenced only the antimicrobial and antimicrobial + insecticide samples.

A second analysis was performed combining all ‘Golden Delicious’ and ‘York’ samples into the same dataset. The classification rate remained similar to the previous results, indicating that differences between apple cultivars did not influence the pre-harvest treatment classification outcome (Table 2S).

3.3.2. Physicochemical + damage discrimination models

The inclusion of the fruit damage evaluation improved the discrimination power of the PCA models. Better separation between the two clusters (control, holistic, and insecticide treatments *versus* antimicrobial and antimicrobial + insecticide treatments) were observed for ‘Golden Delicious’ samples (Figure 4A). There was strong separation of these groups along PC1, with the antimicrobial and antimicrobial + insecticide treatments associated with increasing fruit mass, percent undamaged fruit, and cedar apple rust disease, and decreasing fruit maturity, flyspeck, and sooty blotch (Figure 4B). A better separation was also observed for ‘York’ samples. Patterns were similar to those for ‘Golden Delicious’, however, the antimicrobial and antimicrobial + insecticide treatments were also associated with decreasing plum curculio damage (Figure 4D).

When PLS-DA was used to develop the discrimination models it was possible to get an excellent separation between some pre-harvest treatments (Table 4). A perfect classification (AUC=1.00) was observed in ‘Golden Delicious’ samples from the holistic, antimicrobial and antimicrobial + insecticide treatments, and a value of 0.96 (almost perfect classification) was found for the insecticide treatment (Table 4). Again, the control samples had the worst classification result (AUC=0.81), indicating that the model was not able to clearly distinguish these samples from the others. The sensitivity values for all classes reached 100%, and the best specificity (97%) was observed for the antimicrobial + insecticide treatment. The classification performance for ‘York’ samples was slightly inferior. The antimicrobial treatment had the best classification (AUC=0.87, sensitivity=100%, specificity=80%), and the AUC values decreased for the control (AUC=0.77), insecticide (AUC=0.84), and antimicrobial + insecticide (AUC=0.83) treatments. A sensitivity of

100% was observed for the antimicrobial and antimicrobial + insecticide treatments, indicating that these classes can be clearly differentiated from the others (Table 4). The best specificity (100%) was observed for the holistic treatment.

The main quality parameters associated with the good classification of each pre-harvest treatment can be evaluated based on the discriminant function (DF) and PLS-DA coefficients (Figure 5). Overall, classification of 'Golden Delicious' fruit quality was related to fruit mass, fruit maturity and the number of undamaged fruit (Figure 5B). On the other hand, damage caused by flyspeck, sooty blotch and cedar apple rust had the largest influence on the unsprayed, holistic, and insecticide treatments. However, fruit mass and SSC were also important parameters (Figure 5B). A similar trend was observed for 'York' samples (Figure 5C and 5D).

When 'Golden Delicious' and 'York' samples were combined, the PLS-DA classification performance was inferior (Table 5) compared to the previous attempt with each cultivar separately (Table 4). This indicates that each cultivar responded differently to the pre-harvest treatments. An almost perfect classification (AUC=0.98) was obtained for the antimicrobial + insecticide treatment, but lower accuracy values were observed for the control (AUC=0.83), holistic (AUC=0.88), insecticide (AUC=0.85), and antimicrobial (AUC=0.85) treatments (Table 5). However, a sensitivity of 100% was observed for the insecticide, antimicrobial, and antimicrobial + insecticide treatments. The control (sensitivity=80%, specificity=78%) and holistic samples (sensitivity=80%, specificity=70%) were misclassified (Table 5), but with better performance when compared to the models that used only physicochemical parameters (Table 1S and 2S).

4. Discussion

Pre-harvest spray treatments can affect various quality parameters of fruit, with downstream consequences for market value, agricultural sustainability, and human health. As appearance determines the purchase intention of produce (Vanoli and Buccheri, 2012), the presence of damage caused by diseases and/or insects, even if primarily cosmetic, is an important quality parameter. Other physicochemical parameters are also critical for determining market value and consumer acceptance of apples. This study showed that pre-harvest treatments can impact various aspects of fruit quality, including disease incidence, mass, soluble solid content, and maturity and that the magnitude of these effects varies among apple cultivars.

The presence of blemishes mainly caused by cosmetic diseases (sooty blotch and fly speck) in both cultivars severely impaired the fruit quality, which resulted in a low percentage of undamaged fruits. Overall, better fruit quality was observed when antimicrobials were used as pre-harvest treatments, as the fungicides used were very effective to control sooty blotch and flyspeck (Williamson and Sutton, 2000). Sooty blotch and flyspeck are among the most common diseases of pome fruits in humid temperate growing regions of the world, such as Virginia, USA (Williamson and Sutton, 2000). These diseases are particularly severe in the southeastern USA and are considered of great economic importance as fruit become unsuitable for fresh market due to the reduced fruit quality. The overall quality of untreated fruit and fruit sprayed with holistic and insecticide treatments was severely affected by the presence of these two diseases. Although the infection levels were significantly lower in the holistic treatment (Table 2), these fruits still all had some level of damage (Figure 1). These fruits may be useful for processing, but fruit quality was not satisfactory for the fresh market.

Other damaging agents observed in the orchard, including cedar apple rust and plum curculio, were more minor. The fungicide spray program used in this study was not optimized to control cedar apple rust, as the typical spray during tight cluster was missed and the bloom spray included only captan as a fungicide, which provides only slight protection against rusts (Pfeiffer et al. 2018). Interestingly, the treatment with insecticides did not result in lower damage caused by plum curculio beetles, although Imidan, which was first applied at petal fall, is rated as excellent for control of these insects (Pfeiffer et al. 2018). It is worth noting that we observed limited insect pressure, which may have been unique to this season and/or the result of low insect populations due to a long history of conventional management in this orchard.

Both cultivars produced heavier and bigger fruit when plants were sprayed with antimicrobial or antimicrobial + insecticide treatments. However, the effects on size were more pronounced in ‘York’ apples (Figure 3). Both ‘Golden Delicious’ and ‘York’ cultivars bear medium size to large fruits (Ingle and D’Souza, 2000; Burford, 2013; Ornelas-Paz et al., 2018), yet ‘Golden Delicious’ trees produced heavier and bigger fruit compared to ‘York’ (Figure 3). Hatcher (1995) reported that in infected leaves the overall photosynthesis declines and the transport of photoassimilate is also affected. The infected leaf exports less photoassimilate (Walters and Ayres, 1982) and exports can almost cease as the infection develops (So and Thrower, 1976). Therefore, it is likely that the antimicrobials controlled fungal infection in the canopy and on the fruit surface, allowing the plant to translocate more photoassimilates to fruit and bear heavier and bigger fruits. The same trend was observed by Hutcheon et al. (1986) in ‘Cox’s Orange Pippin’ apple trees sprayed with different fungicides.

The other main fruit quality parameter that was affected by the pre-harvest treatments was the soluble solid content, which was significantly higher in the holistic treatment compared to other treatments. The holistic treatment contained a variety of products, including fish and kelp-based fertilizers and certain microorganisms that might directly benefit the plant host by stimulating plant immune responses and/or acting as biocontrol agents (Song et al., 2012; Phillips, 2012). Past work has shown that foliar fertilization with certain nutrients such as Zn, B, P, and Ca can increase the content of sucrose, glucose, fructose, and sorbitol in apples (Stampar et al. 1999); however, in another study, fertilization with N and Zn decreased soluble solid content (Amiri et al. 2008). Considering the mix of products in the holistic treatment, the mechanism of observed changes in fruits is unclear, but overall this treatment did improve the quality of fruits somewhat relative to the controls by both increasing sugar content of fruit and reducing the severity of damage from disease.

It is important to note that, in this study, the holistic spray was taken out of the context of a larger holistic program in which it is recommended (Phillips 2012) and applied over a single growing season, thus additional or different effects on quality may be seen in a different agroecological context.

Fruit quality parameters also differed strongly between the two cultivars (Table 3). 'Golden Delicious' is an early season cultivar which bears apples with green to yellow skin. On the other hand, 'York' is a late season cultivar with light red blush to full red skin apples (Virginia Apples, 2018). Consequently, differences in fruit quality might be related to these physiological differences, especially fruit maturity. 'Golden Delicious' is an early-season cultivar and was harvested ahead of 'York' apples. Still, 'Golden Delicious' apples were more mature than 'York' at the time of harvest. Therefore, fruits were sweeter, with a higher SSC and a higher starch-iodine index, indicating starch degradation into soluble sugars (Doerflinger et al., 2015). The more advanced maturity of 'Golden Delicious' apples was also confirmed by the higher moisture and lower DM contents. These findings agree with Ornelas-Paz et al. (2018), who also reported increases in moisture content, and consequent DM reduction, in 'Golden Delicious' apples during on-tree development. Fruit firmness was higher than 'York', but were in the range of what is commonly reported for 'Golden Delicious' produced in other regions (Felicetti and Mattheis 2010). On the other hand, as 'York' is a late season cultivar, the fruit were less mature, with lower SSC, starch-iodine index, and moisture content, and higher DM content. Fruit firmness was lower than 'Golden Delicious', though. According to Ingle and D'Souza (2000), due to the local commercial importance of 'York' apple, few publications are available regarding maturation and storage of this cultivar. Our results provide additional information on the factors affecting fruit quality in this cultivar.

The multivariate analysis confirmed the quality differences observed in the univariate analysis and it was possible to obtain a clear separation between pre-harvest treatments, mainly when the fruit damage evaluation was incorporated into the dataset. Using PCA, which is an unsupervised exploratory data analysis (Bro and Smilde, 2014), it was possible to observe the formation of just two clusters representing the main quality differences. On the other hand, using a supervised algorithm PLS-DA (Brereton and Lloyd, 2014) it was possible to improve the discrimination between classes. With this algorithm, apple samples of ‘Golden Delicious’ cultivar could be correctly classified according to the holistic, antimicrobial, and antimicrobial + insecticide treatments, and the proportion of positive samples correctly identified (sensitivity) reached 100% for all pre-harvest treatments. The performance of the PLS-DA models for ‘York’ samples was not as accurate as ‘Golden Delicious’, possibly because we used fewer samples from ‘York’ to develop the models. Therefore, the inclusion of more data into the dataset improved the robustness and increased the classification accuracy.

5. Conclusions

Pre-harvest spray programs affected ‘Golden Delicious’ and ‘York’ apple fruit quality mainly by controlling in-field disease development which ultimately affected fruit mass. Apple trees sprayed with antimicrobial and antimicrobial + insecticide treatments had less damage caused by diseases and produced bigger and heavier fruit compared to unsprayed (control) trees and those treated with holistic and insecticide sprays. The holistic spray also reduced the severity of sooty blotch and flyspeck somewhat relative to controls.

Pre-harvest spray treatments also affected the soluble solids content (SSC) and fruit maturity with the holistic treatment resulting in fruit with higher SSC and the antimicrobial treatment resulting in fruit with lower starch-iodine index.

PCA-LDA and especially PLS-DA were both useful to discriminate fruit quality, but better pre-harvest spray treatment discrimination was achieved using PLS-DA with ‘Golden Delicious’ fruit. Fruits were well classified according to the holistic, antimicrobial, and antimicrobial +

insecticide pre-harvest treatments. Unsprayed and insecticide treated fruit clustered together, indicating similarities between fruit quality, especially for ‘York’ fruit.

These results contribute to a broader understanding of the factors impacting fruit quality in one of the most economically important fruit crops in temperate regions.

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Tables

Table 1. Spray dates and products used as pre-harvest treatments on 'Golden Delicious' and 'York' apple trees* during 2018 season in Blacksburg, Virginia, USA.

Date	**Phenological event**	**Treatment**	**Product(s)**
23 April 2018	Bloom	Untreated	None
		Holistic	Liquid fish, effective microbes, molasses, liquid kelp
		Insecticides	None
		Antimicrobials	Captan®[1] and streptomycin
		Antimicrobials+fungicides	Captan® and streptomycin
07 May 2018	Petal fall	Untreated	None
		Holistic	Neem oil, soap emulsifier, liquid fish, effective microbes, molasses, liquid kelp
		Insecticides	Imidan®[2]
		Antimicrobials	Mancozeb®[3] and Oxytetracycline
		Antimicrobials+fungicides	Imidan®, Mancozeb®, and Oxytetracycline
21 May 2018	First cover	Untreated	None
		Holistic	Neem oil, soap emulsifier, liquid fish, effective microbes, molasses, liquid kelp
		Insecticides	Altacor®[4]
		Antimicrobials	Captan® and streptomycin
		Antimicrobials+fungicides	Altacor®, Captan®, and streptomycin
04 June 2018	Second cover	Untreated	None
		Holistic	Neem oil, soap emulsifier, liquid fish, effective microbes, molasses, liquid kelp
		Insecticides	Imidan®
		Antimicrobials	Mancozeb®, and Oxytetracycline
		Antimicrobials+fungicides	Imidan®, Mancozeb®, and Oxytetracycline
18 June 2018	Third cover	Untreated	None
		Holistic	Neem oil, soap emulsifier,

			liquid fish, effective microbes, molasses, liquid kelp
		Insecticides	Altacor®
		Antimicrobials	Captan® and streptomycin
		Antimicrobials+fungicides	Altacor®, Captan®, and streptomycin
02 July 2018	Fourth cover	Untreated	None
		Holistic	Neem oil, soap emulsifier, liquid fish, effective microbes, molasses, liquid kelp
		Insecticides	Imidan®
		Antimicrobials	Mancozeb®, and Oxytetracycline
		Antimicrobials+fungicides	Imidan®, Mancozeb®, and Oxytetracycline
16 July 2018	Fifth cover	Untreated	None
		Holistic	Neem oil, soap emulsifier, liquid fish, effective microbes, molasses, liquid kelp
		Insecticides	Altacor®
		Antimicrobials	Captan® and streptomycin
		Antimicrobials+fungicides	Altacor®, Captan®, and streptomycin
30 July 2018	Sixth cover	Untreated	None
		Holistic	Neem oil, soap emulsifier, liquid fish, effective microbes, molasses, liquid kelp
		Insecticides	Imidan®
		Antimicrobials	Mancozeb®, and Oxytetracycline
		Antimicrobials+fungicides	Imidan®, Mancozeb®, and Oxytetracycline
13 August 2018	Seventh cover	Untreated	None
		Holistic	Neem oil, soap emulsifier, liquid fish, effective microbes, molasses, liquid kelp
		Insecticides	Altacor®
		Antimicrobials	Captan® and streptomycin
		Antimicrobials+fungicides	Altacor®, Captan®, and streptomycin
27 August 2018	Eighth cover	Untreated	None
		Holistic	Neem oil, soap emulsifier,

			liquid fish, effective microbes, molasses, liquid kelp
		Insecticides	Imidan®
		Antimicrobials	Mancozeb®, and Oxytetracycline
		Antimicrobials+fungicides	Imidan®, Mancozeb®, and Oxytetracycline
10 September 2018	Ninth cover	Untreated	None
		Holistic	Neem oil, soap emulsifier, liquid fish, effective microbes, molasses, liquid kelp
		Insecticides	Altacor®
		Antimicrobials	Captan® and streptomycin
		Antimicrobials+fungicides	Altacor®, Captan®, and streptomycin

[*]Sprays started from bloom to fruit maturity and the whole apple plants were sprayed using a tow behind sprayer. [1]N-(trichloromethylthio) cyclohex-4-ene-1,2-dicarboximide, [2]N-(Mercaptomethyl) phthalimlde, S-(O,Q-dimethyt phosphorodithioat, [3]Manganese ethylenebis(dithiocarbamate) (polymeric) complex with zinc salt, [4]3-Bromo-N-[4-chloro-2-methyl-6-[(methylamino)carbonyl]phenyl]-1-(3-chloro-2-pyridinyl)-1H-pyrazole- 5-carboxamide.

Table 2. Fruit damage evaluation of ‘Golden Delicious’ and ‘York’ apples submitted to different pre-harvest spray treatments during the 2018 growing season in Blacksburg, Virginia, USA.

Parameters	**Flyspeck (0 to 5)[1]**	**Sooty blotch (0 to 5)[2]**	**Cedar apple rust (%)[3]**	**Plum curculio (%)[4]**
Cultivars (C)				
‘Golden Delicious’$_{\text{35 trees, 684 fruits}}$	2.32±1.87 b	2.40±1.78 b	3.86±5.30 b	7.29±8.43
‘York’$_{\text{28 trees, 394 fruits}}$	2.70±1.63 a	3.14±1.71 a	12.11±12.21 a	12.18±18.41
$F_{9,62}$	4.85	47.40	14.15	2.11
p-value	0.0320	<.0001	0.0004	0.1526
Treatments (T)				
Control$_{\text{11 trees, 191 fruits}}$	4.43±0.78 a	4.48±0.60 a	8.91±14.46	14.55±19.39
Holistic$_{\text{12 trees, 176 fruits}}$	3.15±0.64 b	3.47±0.73 b	3.75±7.42	16.42±19.20
Insecticides$_{\text{13 trees, 172 fruits}}$	4.07±1.11 ab	4.64±0.51 ab	8.08±11.24	6.00±9.11
Antimicrobials$_{\text{13 trees, 259 fruits}}$	0.86±0.62 c	0.92±0.53 c	9.31±7.66	7.00±9.37
Insecticides+antimicrobials$_{\text{14 trees, 280 fruits}}$	0.63±0.50 c	0.87±0.61 c	2.50±8.26	5.00±7.34
$F_{9,62}$	84.51	250.60	0.61	2.32
p-value	<.0001	<.0001	0.6553	0.0688
Interaction (C x T)				
$F_{9,62}$	1.88	1.15	2.78	0.79
p-value	0.1280^{NS}	0.3436^{NS}	0.0359^{NS}	0.5360^{NS}
CV (%)	27.82	15.42	114.99	140.56

[1]Mean (±SD) flyspeck (*Zygophiala jamaicensis* Mason) damage rated on a scale from 0 (no damage) to 5 (> 75% coverage of fruit). [2]Mean (±SD) sooty blotch damage rated on a scale from 0 (no damage) to 5 (> 75% coverage of fruit). [3]Mean (±SD) cedar apple rust (*Gymnosporangium juniperi-virginianae*) damage. [4]Mean (±SD) plum curculio (*Conotrachelus nenuphar* Herbst). Values with the same letter within the columns are not statistically different by Tukey’s test (p<0.05). Values in the column without letter are not statistically different by Tukey’s test (p<0.05). NS = not significant. CV = coefficient of variation.

Table 3. Fruit quality parameters of ‘Golden Delicious’ and ‘York’ apples submitted to different pre-harvest spray treatments during the 2018 growing season in Blacksburg, Virginia, USA.

Parameters	**Mass (g)**	**SSC[1] (%)**	**Firmness (N)**	**Maturity[2] (1 to 8)**	**Dry matter (g / kg)**	**Moisture (g / kg)**
Cultivars (C)						
‘Golden Delicious’$_{36\ \text{trees},\ 108\ \text{fruits}}$	110.61±22.80 a	13.72±1.18 a	74.63±7.34 a	7.70±0.52 a	143.5±17.3 a	856.5±17.3 b
‘York’$_{33\ \text{trees},\ 98\ \text{fruits}}$	76.67±40.42 b	11.01±0.84 b	57.74±10.25 b	6.06±1.93 b	124.3±18.4 b	875.6±08.4 a
$F_{9,68}$	78.94	154.48	65.95	28.69	20.80	20.80
p-value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Treatments (T)						
Control$_{13\ \text{trees},\ 38\ \text{fruits}}$	77.93±29.61 b	12.17±1.61 b	69.02±8.69	6.86±1.80 b	130.9±20.3	869.1±20.3
Holistic$_{15\ \text{trees},\ 45\ \text{fruits}}$	75.53±30.49 b	13.16±1.92 a	66.63±13.55	7.62±0.51 ab	142.0±25.0	858.0±25.0
Insecticides$_{14\ \text{trees},\ 42\ \text{fruits}}$	64.50±28.52 b	11.69±1.59 b	67.61±13.53	7.36±1.47 ab	127.3±18.9	872.7±18.9
Antimicrobials$_{13\ \text{trees},\ 39\ \text{fruits}}$	130.31±16.84 a	12.77±1.68 ab	66.19±13.28	5.84±1.80 b	139.1±19.3	860.9±19.3
Insecticides+antimicrobials$_{14\ \text{trees},\ 42\ \text{fruits}}$	122.29±19.81 a	12.00±1.37 b	61.53±11.44	6.60±1.76 b	129.6±13.2	870.3±13.2
$F_{9,68}$	46.19	6.37	1.43	3.92	19.3	19.3
p-value	<.0001	0.0002	0.2365	0.0069	0.1169	0.1169
Interaction						
$F_{9,68}$	<.0001	0.4525^{NS}	0.2274^{NS}	0.3352^{NS}	0.4818^{NS}	0.4818^{NS}
p-value	7.49	0.93	1.46	1.17	0.86	0.86
CV (%)	17.01	7.26	12.97	175.10	129.7	20.1

[1]SSC = soluble solids content expressed as a percentage in solution by mass. [2]Cornell starch-iodine index on a scale from 1 (immature) to 8 (fully mature). Average values with the same letter within the columns are not statistically different by Tukey’s test (p<0.05). Values in the column without letter are not statistically different by Tukey’s test (p<0.05). NS = not significant. CV = coefficient of variation.

Table 4. PLS-DA results for discriminating the pre-harvest treatments five groups (1, control; 2, holistic; 3, insecticide; 4, antimicrobials; and 5, antimicrobial + insecticides treatments) of apple samples from two cultivars ('Golden Delicious' and 'York') based on the physicochemical parameters and fruit damage evaluation (flyspeck, sooty blotch, undamaged, plum curculio, cedar apple rust) separately by cultivar.

	'Golden Delicious'					**'York'**				
Accuracy	1	2	3	4	5	1	2	3	4	5
Training (%)	91	83	91	94	91	96	96	100	96	74
Cross-validation (%)	63	77	69	66	91	42	63	57	42	69
Test (%)	88	92	88	92	96	57	50	73	90	85
AUC[1]	0.81	1.00	0.96	1.00	1.00	0.77	0.59	0.84	0.87	0.83
Sensitivity (%)	100	100	100	100	100	50	0	67	100	100
Specificity (%)	75	83	75	83	92	64	100	80	80	70

[1]AUC = area under the curve.

Table 5. PLS-DA results for discriminating the preharvest treatments five groups (1, control; 2, holistic; 3, insecticide; 4, antimicrobials; and 5, antimicrobial + insecticides treatments) of apple samples from two cultivars ('Golden Delicious' and 'York') based on the physicochemical parameters and fruit damage evaluation (flyspeck, sooty blotch, undamaged, plum curculio, cedar apple rust) combining the two cultivars.

	'Golden Delicious' + 'York'				
Accuracy	1	2	3	4	5
Training (%)	73	70	84	89	91
Cross-validation (%)	76	70	65	75	70
Test (%)	79	75	84	86	89
AUC[1]	0.83	0.88	0.85	0.86	0.98
Sensitivity (%)	80	80	100	100	100
Specificity (%)	78	70	68	73	77

[1]AUC = area under the curve.

Figures

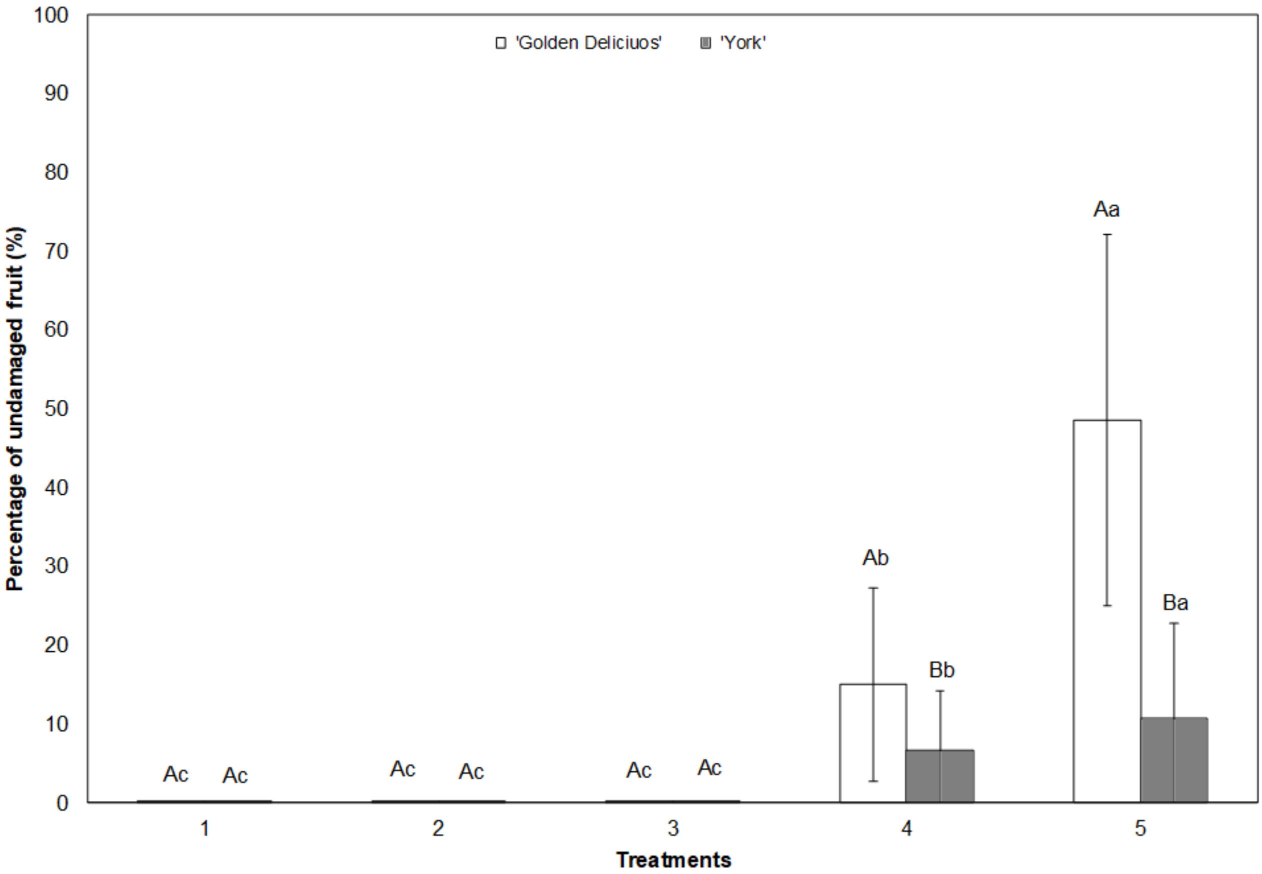

Figure 1. Percentage of undamaged fruit (%) of ‘Golden Delicious’ (GD) and ‘York’ (Y) submitted to different pre-harvest treatments: 1, control – unsprayed trees (7 trees, 133 fruits GD; 4 trees, 57 fruits Y), 2, trees sprayed with holistic (8 trees, 161 fruits GD; 4 trees, 16 fruits Y), 3, trees sprayed with insecticides (6 trees, 110 fruits GD; 7 trees, 62 fruits Y), 4, trees sprayed with antimicrobials (7 trees, 140 fruits GD; 6 trees, 119 fruits Y), and 5, trees sprayed with antimicrobials + insecticides (7 trees, 140 fruits GD; 7 trees, 140 fruits Y). Treatments with the same lowercase letters are not statistically different by Tukey’s test ($p<0.05$) within cultivars. Cultivars with the same capital letters are not statistically different by Tukey’s test ($p<0.05$) within treatments. The bars represent the standard deviation of each plant repetitions.

Figure 2. ‘Golden Delicious’ (top) and ‘York’ (bottom) apples submitted to different pre-harvest treatments: 1, control – unsprayed trees, 2, trees sprayed with holistic, 3, trees sprayed with insecticides, 4, trees sprayed with antimicrobials, and 5, trees sprayed with antimicrobials + insecticides.

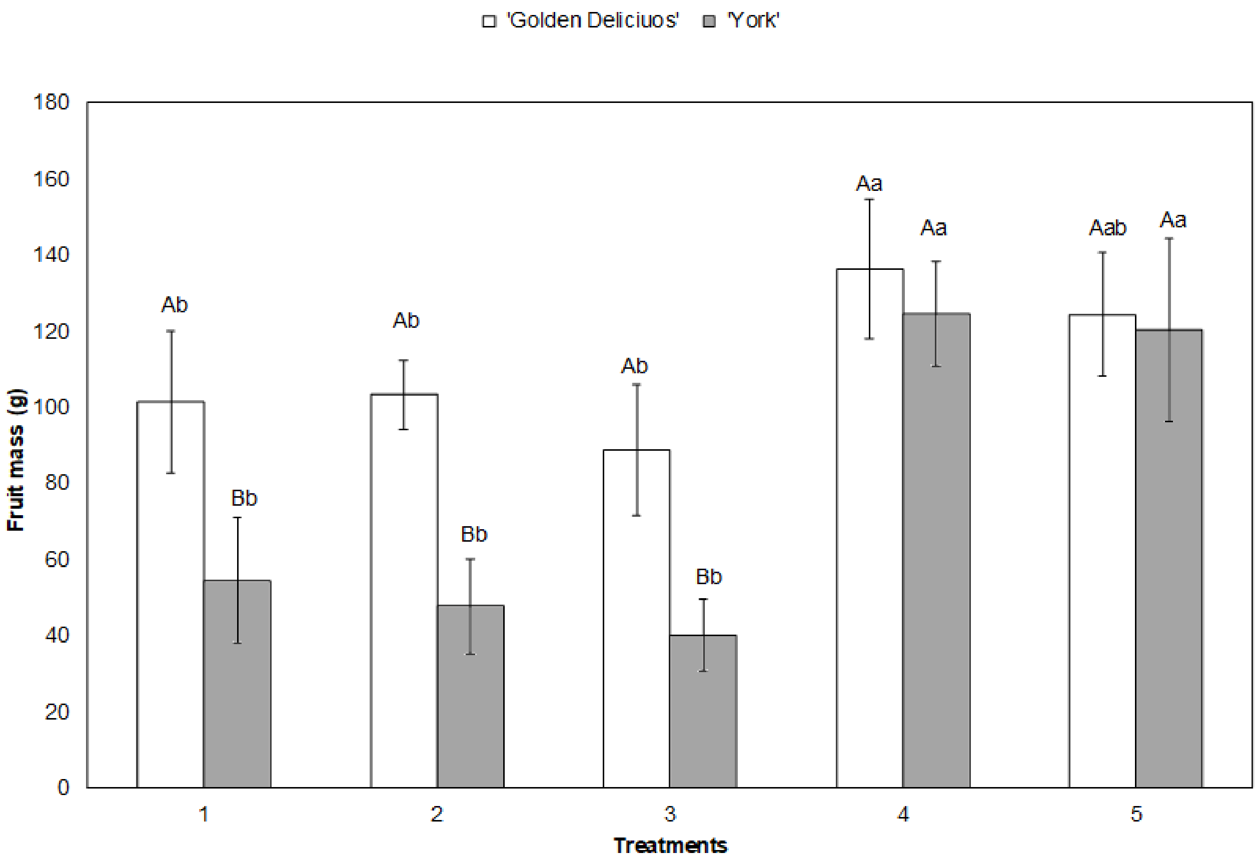

Figure 3. Fruit mass (g) of 'Golden Delicious' and 'York' submitted to different pre-harvest treatments: 1, control – unsprayed trees (7 trees, 21 fruits GD; 6 trees, 17 fruits Y), 2, trees sprayed with holistic (8 trees, 24 fruits GD; 7 trees, 21 fruits Y), 3, trees sprayed with insecticides (7 trees, 21 fruits GD; 7 trees, 21 fruits Y), 4, trees sprayed with antimicrobials (7 trees, 21 fruits GD; 6 trees, 18 fruits Y), and 5, trees sprayed with antimicrobials + insecticides (7 trees, 21 fruits GD; 7 trees, 21 fruits Y). Treatments with the same lowercase letters are not statistically different by Tukey's test ($p<0.05$) within cultivars. Cultivars with the same capital letters are not statistically different by Tukey's test ($p<0.05$) within treatments. The bars represent the standard deviation of each plant repetitions.

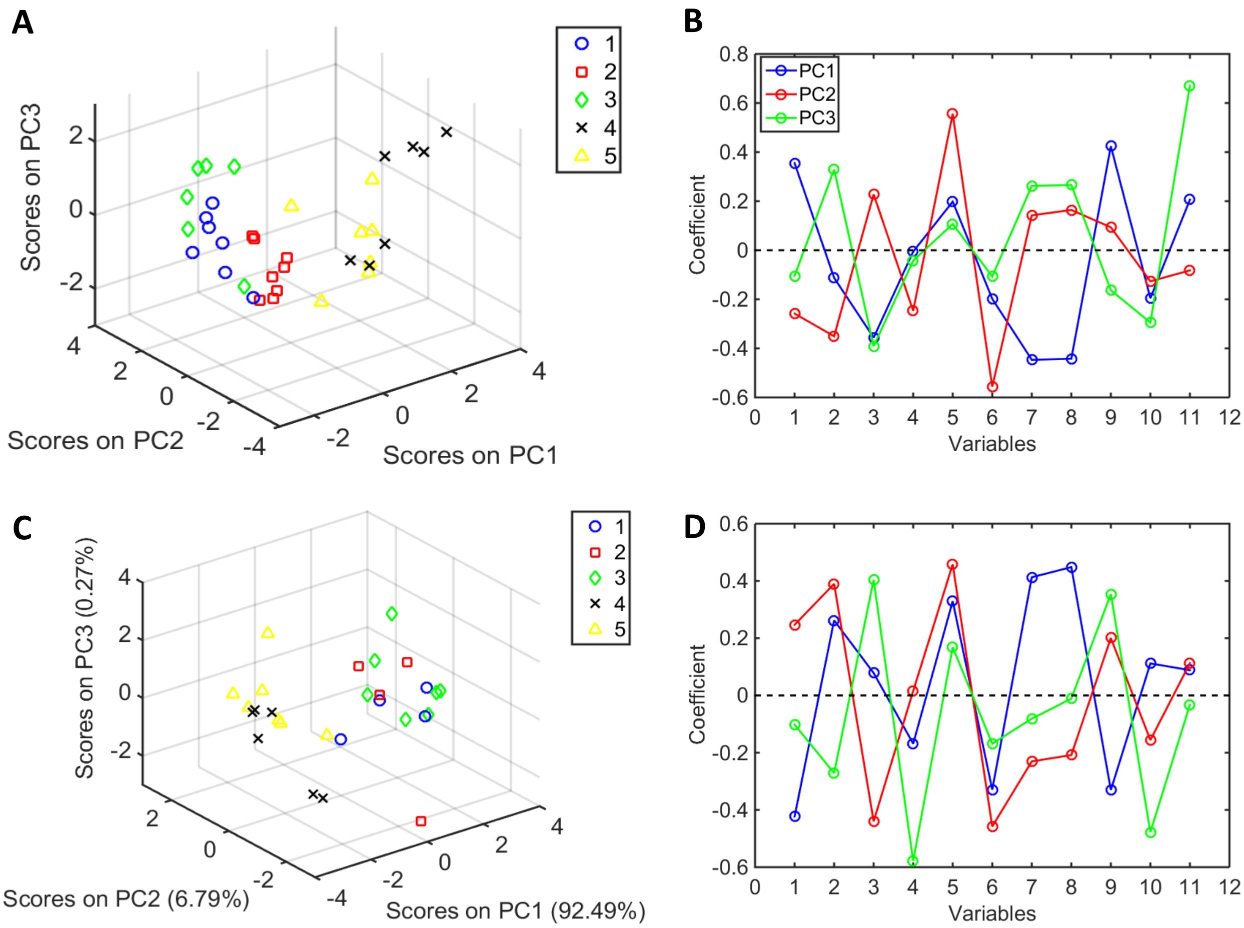

Figure 4. PCA scores (A) and loadings (B) for 'Golden Delicious' and PCA scores (C) and loadings (D) for 'York' cultivars. Class legend (A & C): 1, control; 2, holistic; 3, insecticide; 4, antimicrobials; and 5, antimicrobial + insecticides treatments. Variables legend (B & D): 1, fruit mass; 2, firmness; 3, maturity; 4, soluble solids content; 5, moisture; 6, dry matter; 7, flyspeck level; 8, sooty blotch level; 9, undamaged; 10, plum curculio; and 11, cedar apple rust.

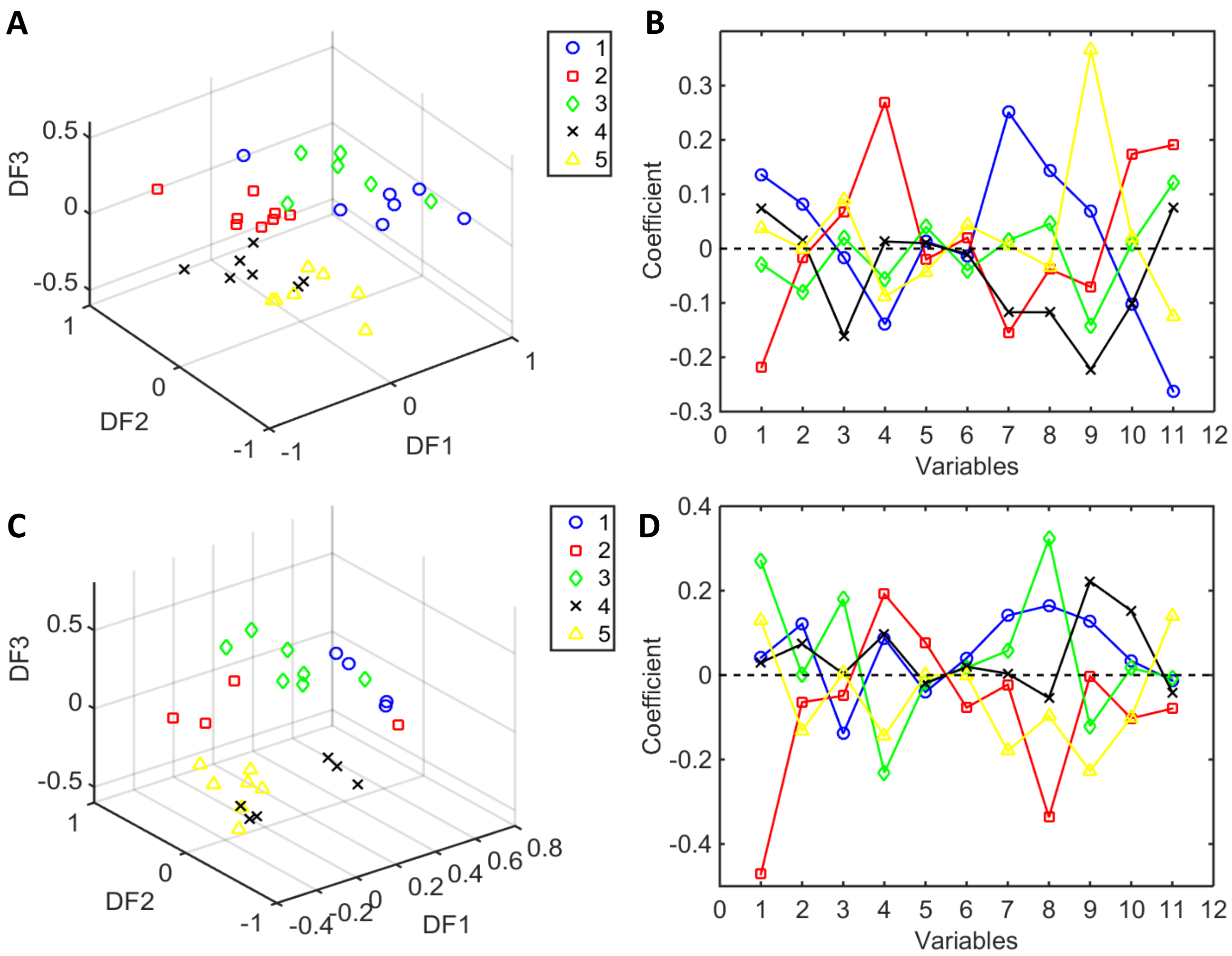

Figure 5. Discriminant function (DF) represented by the predicted PLS-DA categories (A) and PLS-DA coefficients for 'Golden Delicious' (B). DF represented by the predicted PLS-DA categories (C) and PLS-DA coefficients for 'York' (D) cultivars. Class legend (A & C): 1, control; 2, holistic; 3, insecticide; 4, antimicrobials; and 5, antimicrobial + insecticides treatments. Variables legend (B & D): 1, fruit mass; 2, firmness; 3, maturity; 4, soluble solids content; 5, moisture; 6, dry matter; 7, flyspeck level; 8, sooty blotch level; 9, undamaged; 10, plum curculio; and 11, cedar apple rust.